Purification and Characterization of a Highly Stable Cysteine Protease from the Latex of *Ervatamia coronaria*

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A highly stable cysteine protease was purified to homogeneity from the latex of *Ervatamia coronaria* by a simple purification procedure involving ammonium carbonate precipitation and ion-exchange chromatography. The molecular mass was estimated to be approximately 25,000 Da by SDS-PAGE and gel filtration. The extinction coefficient (ε<sub>280</sub>) of the enzyme was 24.6. The enzyme hydrolyzed denatured natural substrates like casein, hemoglobin, azoalbumin, and azocasein with a high specific activity but showed low specific activity towards synthetic substrates. The pH and temperature optima were 7.5–8.0 and 50°C respectively. The activity of the enzyme was strongly inhibited by thiol-specific inhibitors like leupeptin, iodoacetamide, PCMB, NEM, and mercuric chloride. The striking property of this enzyme was its stability over a wide pH range (2–12) and other extreme conditions of temperature, denaturants, and organic solvents. The N-terminus sequence showed marked similarity to known cysteine proteases.

**Key words:** plant latex; cysteine protease; plant endopeptidase; *Ervatamia coronaria*; ervatamin

*Ervatamia coronaria*, a flowering plant (Family: *Apocynaceae*) indigenous to India, has a wide range of medicinally important applications. The latex is cooling and has an anti-inflammatory effect on wounds. Roots rubbed into a paste work as a vermicide. Different parts of the plant have a variety of other applications. The latex of this plant has been shown to contain a few medicinally valuable alkaloids. However, so far there is no report regarding the biochemical constituents of the latex. It was important and interesting to look into the biochemical components of the latex of this plant in view of its applications. In the course of screening test of proteases, the latex of this plant showed cascinolytic activity and encouraged us to look into it in detail. In this paper, isolation, characterization, and substrate specificity of a protease from the latex of *Ervatamia coronaria* are reported. It is a cysteine protease and according to the well known cysteine protease nomenclature it has been named ervatamin.

**Materials and Methods**

**Materials.** Fresh latex was obtained from young stems of the plant *Ervatamia coronaria*. SP-Sepharose was purchased from Pharmacia. BSA, RNase A, hen egg white lysozyme, azocasein, azoalbumin, hemoglobin, DTNB, DTT, GuHCl, urea, o-phenanthroline, EDTA, EGTA, leupeptin, SBTI, NEM, β-mercaptoethanol, PMSF, hemoglobin, Coomassie brilliant blue R 250, all synthetic substrates, and other standard proteins were obtained from Sigma Chemical Co., USA. Coomassie brilliant blue G250 was from Eastman Kodak. TFA was obtained from Applied Biosystems. Acetonitrile was of HPLC grade. All other chemicals were of highest purity available commercially. Sodium tetraphosphate (Na<sub>4</sub>S<sub>2</sub>O<sub>7</sub>·2H<sub>2</sub>O) was synthesized by the method of Gilman et al. Papain was purified from dried latex purchased from Enzochem, India, by the method of Kimmel and Smith. Hemoglobin was denatured with urea before assay as described.

**Purification of the protease.** All purification steps were done at 4°C unless specified otherwise.

**Step 1-Removal of gum.** Latex was collected from young stems in 650 ml of 0.01 Na-phosphate buffer, pH 7.0, containing sodium tetraphosphate, and frozen at −20°C for 24 h. The latex was thawed to room temperature and centrifuged at 24,000 × g for 15 min to remove any insoluble material. The supernatant, free of gum and other debris, was dialyzed against 0.01 M phosphate buffer, pH 7.0. The resulting supernatant was collected by centrifugation at 24,000 × g for 15 min.

**Step 2-Ammonium sulfate precipitation.** Ammonium sulfate was added to the supernatant to 70% saturation. In this step the total protein concentration was maintained around 0.6 mg per ml. The precipitation was allowed for 15 h and the precipitate was collected by centrifugation at 24000 × g for 15 min. The collected precipitate was dissolved in 0.01 M acetate buffer, pH 5.0, and dialyzed against the same buffer for 24 h with frequent changes of the buffer. Any insoluble material

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Abbreviations: PCMB, p-chloromercuribenzoate; NEM, N-ethyl maleimide; GuHCl, Guanidine hydrochloride; DTNB, 5,5'-dithiobis-(2-nitro benzoic acid); DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol bis(β-amino ethyl ether) N,N,N',N''-tetraacetic acid; SBTI, soybean trypsin inhibitor; PMSF, phenylmethanesulfonyl fluoride; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; BAPA, benzoyl arginine p-nitroanilide.
in the dialysis bag was removed by centrifugation as above.

**Step 3-Cation exchange chromatography.** Protein solution from the above step was put on a SP-Sepharose fast flow ion exchange column that was pre-equilibrated with 0.01 M acetate buffer, pH 5.0. The column was thoroughly washed with the same buffer and protein was eluted with a linear salt gradient of 0–1 M NaCl. Fractions of 5 ml volume were collected at a flow rate of 6 ml/min. The fractions were monitored by absorbance at 280 nm and their protein was measured by Bradford assay. The caseinolytic activity and purity of each fraction were assessed. Fractions with maximum activity and homogeneity were pooled and concentrated by 80% ammonium sulfate saturation followed by dialysis against 0.01 M Na-phosphate buffer, pH 7.0. The clear protein solution was filtered to sterility and stored at 4°C for further use.

Sodium tetraphionate was used throughout the purification procedure to avoid complication due to any autodigestion.

**Protein concentration.** Protein concentration was measured spectrophotometrically (A$_{280}$) as well as by the Bradford assay using BSA as standard.

**Assay for enzyme activity.** The hydrolyzing activity of the enzyme was monitored using denatured substrates like casein, hemoglobin, azoalbumin, and azocasein. The proteolytic activity of the enzyme was followed by the method of Arnon. Before the assay the tetraphionate-inactivated enzyme (5–10 µg) was activated at 37°C by 0.05 M β-mercaptoethanol in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.02 M EDTA for 15 min, in a volume of 0.5 ml. In each case, 0.5 ml of 6–2% (w/v) substrate in the same buffer was added and the reaction was allowed to proceed for 30 min at 37°C. The reaction was stopped by an addition of 0.2 ml of 10% TCA. After the solution stood for 10 min at room temperature, the precipitate was removed by centrifugation and the absorbance of the TCA soluble peptides was measured at 280 nm. The control assay was done without any enzyme in the reaction mixture and used as a reference. When azoalbumin and azocasein were used as substrates, the resulting supernatant obtained after TCA precipitation was mixed with an equal volume of 0.5 M NaOH. After 15 min the color developed was measured by absorbance at 440 nm. One unit of enzyme activity is defined as the amount of enzyme which, under the assay conditions described, gives rise to an increase of one unit of absorbancy at 280 nm or 440 nm per minute of digestion. The specific activity is the number of units of activity per milligram of protein.

**Assay for proteolytic activity towards peptidyl-pNA.** The enzymatic hydrolysis of peptidyl-pNA substrates by the purified protease was studied by spectrophotometry. In each case 1 mM solution of the synthetic substrate was prepared by dissolving the required amount of the substrate in DMSO and making up the final volume with buffer of desired pH at a temperature greater than 25°C. Before the assay the enzyme was activated as described above. To 10–50 µg of active enzyme in 0.5 ml of buffer, 1 ml of the synthetic substrate was added and digestion was allowed to proceed for 30 min at 37°C. The reaction was stopped by addition of 0.2 ml of 30% acetic acid. The liberated para nitroaniline was measured spectrophotometrically at 410 nm using an extinction coefficient of 8,800 M$^{-1}$ cm$^{-1}$ as a measure of the hydrolysis.

**Effects of pH on the enzyme activity.** The effects of pH, in the range of 1–11, on the activity of the purified enzyme were investigated using natural substrates. The buffers used were: 0.05 M KCl–HCl (pH 1.0–1.5); 0.05 M glycine-HCl (pH 2.0–3.5); 0.05 M acetate (pH 4.0–5.5); 0.05 M phosphate (pH 6.0–7.5), 0.05 M Tris-HCl (pH 8.0–10.0) and 0.05 M carbonate (pH 10.5–11.0). Solutions of azoalbumin and hemoglobin were prepared in these buffers. The enzyme was equilibrated in 0.5 ml buffer of given pH for 15 min in the presence of an activator at 37°C and was assayed at the same pH as described above. Enzyme assay below pH 4.0 could not be done with azoalbumin as substrate due to its insolubility. However, the enzyme activity was checked below pH 4.0 using denatured hemoglobin.

**Temperature optima.** The temperature optimum for the proteolytic activity of the purified enzyme was identified using azoalbumin at specified temperatures as substrate. The enzyme was equilibrated in 0.05 M Tris-HCl, pH 8.0 at different temperatures in the range of 15°C to 80°C for 15 min. A sample was used for the assay at the same temperature. The assay was done by the method described above.

**Effects of activators on enzymatic activity.** The effects of various activators on the proteolytic activity of the enzyme were measured. Activators used were thiolspecific like β-mercaptoethanol, DTT, cysteine, and glutathione. The concentrations of the enzyme and activator were 5 µg/ml and in the range 0-40 mM respectively. The enzyme was incubated for 15 min with the activator and a sample was used for the assay. The assay was done by the method described above.

**Effects of inhibitors on enzymatic activity.** Both thiol-specific and nonspecific inhibitors were used to check the inhibition of the hydrolysis of a substrate by the enzyme. The tetraphionate-inactivated enzyme was activated by 0.05 M β-mercaptoethanol at room temperature in 0.05 M Tris-HCl buffer, pH 8.0 containing 0.002 M EDTA for 15 min. The activator was removed subsequently by dialysis against 0.1 M acetic acid at 4°C with frequent changes of the dialysate. In each case, 1 µM of enzyme was incubated in presence of increasing concentrations of inhibitor in 0.05 M Tris-HCl buffer, pH 8.0 for 20 min at 37°C and assayed with azoalbumin as a substrate. The inhibitors used were sodium tetrathionate, iodoacetamide, PMSF, mercuric chloride, PCMB, leupeptin, EDTA, EGTA, SBTI, o-
phenanthroline, and NEM. A control assay was done with enzyme solution without inhibitors and the resulting activity was considered as 100%.

Effects of substrate concentration on reaction velocity. The effects of increasing substrate concentration on the reaction velocity were studied using azoalbumin as a substrate at the optimum pH. In each assay 5 μg of the enzyme was used and the substrate concentration was increased from 0–6 μM. A Lineweaver-Burk plot was plotted and the value of the Michaelis-Menten constant ($K_m$) was calculated.

SDS-polyacrylamide gel electrophoresis. Assessment of homogeneity and estimation of molecular mass ($M_r$) of the purified ervatamin were done on 15% SDS-PAGE under reducing conditions at room temperature by the method of Laemmli. Samples of active and irreversibly inactivated enzyme were put on the gel. For molecular mass measurement, ovo transferrin (76 kDa), albumin (66.25 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (30 kDa), myoglobin (17.2 kDa), and cytochrome c (12.3 kDa) were used as standards. The proteins were stained with Coomassie brilliant blue R-250.

Gel filtration. Molecular mass ($M_r$) of the purified enzyme was estimated by gel filtration as described by Fish et al. on a TSK G2000SW (from LKB) gel filtration column fitted to a LKB Bromma HPLC system provided with a variable wavelength detector. Fully reduced and blocked protein behaves as a random polypeptide chain and its elution volume is a function of its molecular weight regardless of the shape the native protein might have. The purified enzyme as well as standard proteins were reduced for 10 h. in 0.05 M Tris-HCl buffer, pH 8.0, containing 6 mM GuHCl, 0.001 M EDTA, and 0.1 M β-mercaptoethanol. After complete reduction, the free sulfhydryl groups were blocked with an excess of iodoacetamide for one hour at room temperature. The reduced and blocked protein was put on a TSK G2000SW gel filtration column equilibrated with 0.05 M phosphate buffer, pH 7.0, and eluted isocratically with the same buffer at a flow rate of 0.5 ml/min while monitoring the eluant at 220 nm. Glyceraldehyde dehydrogenase (36 kDa), carbonic anhydrase (30 kDa), papain (23.4 kDa), SBTI (20.1 kDa) and lysozyme (14.4 kDa) were used as molecular mass standards. The void volume ($V_0$) was measured using blue dextran and $V_e$ was the elution volume of the standards as well as ervatamin.

Calculation of extinction coefficient. The extinction coefficient of ervatamin was calculated by dry weight and spectrophotometric methods. From a stock solution of purified enzyme in water several solutions were prepared by serial dilution. The absorbance of these solutions was measured at 280 nm and the samples were dried in an oven at 108–110°C. The dry weight of the protein in each sample was measured using an analytical balance. The extinction coefficient was calculated using Beer Lambert's law, $ε_{280} = \frac{Acl}{c \cdot a}$ (where $A$, absorbance at 280 nm; $c$, concentration of protein in mg/ml and $l$, path length of the cuvette). In the spectrophotometric method, the extinction coefficient was calculated from the formula $ε_{280} = \frac{10(5690n_a + 1280n_b + 120n_c)}{M}$ where $n_a$, $n_b$, and $n_c$ are the number of tryptophan, tyrosine, and cysteine residues in the protein; $M$ is the molecular weight of the protein and 5690, 1280, and 120 are the respective extinction coefficients of tryptophan, tyrosine, and cysteine residues. The numbers of these residues were counted as described below.

Tyrosine and tryptophan content. The tyrosine and tryptophan contents of the enzyme were measured spectrophotometrically using the method of Goodwin and Morton. The absorbance spectra of the enzyme in 0.1 M NaOH was recorded between 300–220 nm using a Beckman DU 640 B spectrophotometer and the absorbance values at 280 nm and 294.4 nm were deduced from the spectra. The following formula was used: $w = (A_{290} - x \cdot e_y)/(e_y - e_x)$ where $w$ is estimated tryptophan content in moles per liter; $A_{290}$ was absorbance at 280 nm from the protein spectra; $e_x$ & $e_y$ were molar extinction coefficients of tyrosine and tryptophan in 0.1 M alkali at 280 nm. ($e_x = 1576$ and $e_y = 5225$). The total tyrosine and tryptophan content in the protein $x$ was calculated using $ε_{294.4} = 2375$. To validate these measurements of tyrosine and tryptophan contents of ervatamin, similar contents of papain, ribonuclease, and lysozyme were also calculated.

Measurement of free and total sulfhydryl content. The free and total sulfhydryl contents of the purified enzyme were measured by the method of Ellman using DTNB, in which the release of nitrothiobenzoate was measured by following the increase in absorbance at 412 nm. The molar extinction coefficient of the TNB anion at 412 nm was 14150 M$^{-1}$ cm$^{-1}$. For free sulfhydryl group measurement, the purified enzyme was activated with 0.01 M β-mercaptoethanol in 0.05 M Tris-HCl, pH 8.0, for 15 min, then dialyzed against 0.1 M acetic acid. Similarly, for the measurement of total sulfhydryl content the enzyme was reduced in the presence of 6 M GuHCl. The free cysteine and total sulfhydryl contents were measured by the DTNB reaction. To validate these measurements, similar contents of papain, ribonuclease, and lysozyme were also measured.

Measurement of carbohydrate content. It is reported that some plant proteases like calotropin FI and FII and stem bromelain are glycoproteins. As ervatamin is also a plant protease, the carbohydrate content of the enzyme was checked by phenol-sulfuric acid method. A standard plot was generated with known concentrations of galactose and the unknown values of the sample were extrapolated. A solution of the enzyme in the concentration range of 1 μg to 10 μg in a volume of 10 μl was taken in a microtiter plate. To each well 25 μl of 4% aqueous phenol was added. After 5 minutes 200 μl of H$_2$SO$_4$ was added and the increase in absorbance was measured at 492 nm.
Autodigestion. Autodigestion of the enzyme, ervatamin, was studied at neutral pH in the presence of an activator, \(\beta\)-mercaptoethanol. The enzyme concentration was in the range of 0.01–0.6 mg/ml. Enzyme at given concentration was incubated at 37°C in 0.05 m Tris-HCl buffer, pH 8.0, containing 0.002 m EDTA. Samples were withdrawn from the incubation mixture at appropriate times and assayed with azoalbunin as substrate. Similarly, samples of tetrathionate-inhibited enzyme were also incubated without an activator and activated for 5 min before the assay. The activity of enzyme in the first five minutes of incubation is used as 100%.

Stability studies. The ability of the purified enzyme to retain its activity under various conditions such as extreme pH, strong denaturants, temperatures, and organic solvents was studied by exposing the enzyme to those conditions. The remaining proteolytic activity of the enzyme was measured at 37°C with azoalbunin as substrate as described previously. The enzyme was incubated in buffers of various pH ranging from 0.5 to 12.0 for 24 h and more. As most of the enzymes are inactivated at higher concentration of GuHCl, urea, SDS, acetonitrile, methanol, ethanol, and at higher temperatures, the extent of stability of ervatamin was monitored under the mentioned conditions. In the case of denaturants and organic solvents, the purified enzyme was incubated for 24 h or more. At different times samples were taken and assayed with azoalbunin as substrate. Similarly the enzyme was incubated at different temperatures for 15 min and assayed at 37°C. The assay was done by the method described above.

N-terminal sequence. The purified enzyme was put on a C18 reverse phase Toypearl HPLC column (7.5 × 300 mm) and eluted with a linear gradient of acetonitrile (0–60% in 0.1% TFA (v/v) in water) for 1 h at flow rate of 1 ml/min using LKB Bromma HPLC system. One-milliliter fractions were collected while monitoring the elution at 280 nm using a variable wavelength detector. The concentration and homogeneity of the fractions were determined spectrophotometrically and on SDS-PAGE respectively. Homogeneous fractions were pooled, dried by speed vac, and sequenced on an Applied Biosystems 477A protein sequencer.

Results

Purification

Chromatography of crude extract of the latex, after ammonium sulfate precipitation, on a cation exchange column gave four peaks (Fig. 1). No proteolytic activity towards casein was found in the unbound eluant. Activity measurements showed that around 90% of the total activity put on was eluted in the fourth eluant (B). Some proteolytic activity was also found in the third peak (A). Both fractions (A and B) were inactivated with sodium tetrathionate. From the preliminary experiments, no significant differences could be detected between these fractions using criteria such as pH and temperature of maximum activity and the sensitivity to various compounds (data not shown). It is suggested that the proteases may be isozymes of each other as reported in stem bromelain.\(^2\) The fractions of the fourth peak (B) that were active and homogeneous were pooled and concentrated. Homogeneity and intactness of the enzyme in the pool was judged on SDS-PAGE as shown in Fig. 2. Two kinds of samples, active and inactive (irreversibly blocked by carboxymethylation to avoid any autolysis) were assessed on the gel. Both active (Lane 2) and inactive samples (Lane 3) resulted in single bands without any significant differences in their mobility. The homogeneity was further confirmed by gel filtration on HPLC using a TSK G2000SW column which had a single symmetrical peak (data not shown). The purification protocol yielded about 10% of the total protein in the extract. The purification results are summarized in Table 1.

Physical Properties

The purified protein showed a single band in SDS-PAGE both in active and inactive forms with an estimated molecular mass \((M_r)\) of 25,500 Da (Fig. 3A) while the molecular mass obtained by gel filtration was 24550 Da (Fig. 3B). The extinction coefficient measured by dry weight and spectrophotometric methods was 24.6 and 23.98 respectively. The values from the two methods were slightly different, which was the case with papain also. Under similar conditions papain yielded values of 25\(^{13}\) and 22.9. Like most cysteine proteases, ervatamin was found to contain no detectable carbohydrate.

Substrate specificity

The enzyme hydrolyzed denatured natural substrates
Fig. 2. SDS-PAGE of Purified Enzyme.
The samples (15 µg) were electrophoresed in 15% polyacrylamide gel under reducing conditions. Lane 1, crude, Lane 2, reversibly active enzyme, Lane 3, inactive enzyme (irreversibly blocked), Lane 4, protein markers: ovotransferrin (76,000), albumin (66,250), ovalbumin (42,700), carbonic anhydrase (30,000), myoglobin (17,200), cytochrome c (12,300) were used as standards. The proteins were stained with Coomassie brilliant blue R-250.

Table 1. Purification of Protease from Ervatamia coronaria

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units*)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>376</td>
<td>12820</td>
<td>36</td>
<td>100</td>
</tr>
<tr>
<td>2. Ammonium Sulfate</td>
<td>160</td>
<td>6338</td>
<td>40</td>
<td>49</td>
</tr>
<tr>
<td>precipitation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. SP-Sepharose FF</td>
<td>36</td>
<td>1239</td>
<td>100</td>
<td>10</td>
</tr>
</tbody>
</table>

* Definition of unit: One unit of enzyme activity is defined as the amount of enzyme that gives rise, under defined assay conditions, to an increase of one unit of absorbancy at 280 nm per minute of digestion.

Fig. 3. Estimation of Molecular Weight of Ervatamin by SDS-PAGE (A) and Gel Filtration (B).
(A) Purified ervatamin (15 µg) was electrophoresed in 15% polyacrylamide gel under reducing conditions. Marker proteins were 1, ovotransferrin (76,000); 2, albumin (66,250); 3, ovalbumin (42,700); 4, carbonic anhydrase (30,000); 5, myoglobin (17,200), 6, cytochrome c (12,300). The closed square (●) was for ervatamin. (B) Reduced and blocked ervatamin was put on a TSK G2000SW column on HPLC and eluted isocratically with 0.05 M phosphate buffer, pH 7.0. Marker proteins were: glyceraldehyde dehydrogenase (36,000) carbonic anhydrase (30,000), papain (23,400) SBTI (20,100), and lysozyme (14, 400). The void volume (V0) was measured using blue dextran and Vt was the elution volume of the standards as well as ervatamin. The closed square (●) was for ervatamin.

like casein, hemoglobin, azoalbumin, and azocasein with high specific activity though it showed very low specific activity or no activity towards synthetic substrates like BAPA, L-Ala Ala p-nitroanilide, succinyl-Ala Ala Ala p-nitroanilide, succinyl phenylalanine p-nitroanilide, L-glutamyl p-nitroanilide and L-alanine p-nitroanilide. However, the purified enzyme showed some activity at very high enzyme concentrations (500-600 µg/ml) with BAPA as a substrate after 2 h of digestion at 37°C. The need of such high concentration of enzyme was reported in the cases of bromelain and actinidin. The exact reason for such low activity of the enzyme towards synthetic substrates is not known.

**pH and temperature optima**
Maximum activity of the enzyme was observed from pH 7.5-8.0 as shown in Fig. 4A and no activity could be detected below pH 5.0. Half maximum activity was obtained at pH 6.5 and 10.5. The hydrolysis of the substrate by ervatamin was optimal around 50°C as shown in Fig. 5A and half maximum activity was found at 30°C and 68°C.

**Effects of activators**
Relative activity of the enzyme at different concentrations of thiol-specific activators such as cysteine, β-mer-
captoethanol, DTT, and glutathione was investigated (data not shown). It appears that at least 0.02 M concentration of the activator is necessary to show the maximum activity under the given conditions. In the case of DTT, however, a little lower concentration was enough to result in the same activation.

**Effects of inhibitors**

Relative activity of the enzyme in presence of different compounds is shown in Table 2. The minimum amount of effective inhibitor required for maximum inhibition has been reported. The activity was inhibited considerably by all the thiol-specific inhibitors studied. PMSF inhibited the activity to some extent though it is a serine-specific inhibitor. Similar observation was reported in the case of papain. The enzyme activity was not inhibited by EDTA and SBTI.

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### Table 2. Effects of Various Compounds on The Proteolytic Activity of Ervatamin towards Denatured Natural Substrates

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration</th>
<th>Relative activity* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>PMSF</td>
<td>40 μM</td>
<td>70</td>
</tr>
<tr>
<td>NEM</td>
<td>20 μM</td>
<td>16.5</td>
</tr>
<tr>
<td>PCMB</td>
<td>6 μM</td>
<td>16.8</td>
</tr>
<tr>
<td>SBTI</td>
<td>100 μg/ml</td>
<td>95</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
<td>100</td>
</tr>
<tr>
<td>EGTA</td>
<td>1 mM</td>
<td>100</td>
</tr>
<tr>
<td>o-Phenanthroline</td>
<td>1 mM</td>
<td>100</td>
</tr>
<tr>
<td>Mercuro chloride</td>
<td>6 μM</td>
<td>10.5</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>40 μM</td>
<td>9.3</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>10 μM</td>
<td>25</td>
</tr>
<tr>
<td>Sodium tetrathionate</td>
<td>20 μM</td>
<td>15.7</td>
</tr>
</tbody>
</table>

* Enzyme activity was measured as described in Materials and Methods.
**Effects of substrate concentration on reaction velocity**

The effects of increasing substrate concentration on reaction velocity follow the typical Michaelis-Menten equation and the apparent $K_m$ obtained from the Lineweaver-Burk plot was $9.09 \times 10^{-9}$ M (data not shown).

**Autodigestion**

Like other proteases, ervatamin also undergoes autodigestion in a solution of neutral pH in the presence of an activator. Figure 6 shows a typical result of loss of activity of the enzyme in the concentration range 0.01–0.6 mg/ml after six hours of incubation. The extent of such loss in activity decreases with an increase in the concentration as seen and no further loss in activity was found above 0.5 mg/ml of the enzyme concentration. No autodigestion was observed even after prolonged incubation at higher concentrations of the enzyme while maximum reduction in the relative activity at the lowest concentration studied (0.01 mg/ml) was less than 40%. At the same time, reversibly inactivated enzyme incubated for the same period of time, in absence of an activator, did not show any loss in activity. This loss in proteolytic activity can be attributed to the autocatalytic properties of the enzyme.

**Specific amino acid residues**

The total sulfhydryl content of the protein was found to be 7 (measured value 6.46) with one free (measured value 1.03) cysteine and the other six forming 3 disulphides. The tyrosine and tryptophan contents of the protein were 16 (measured value 16.13) and 7 (measured value 7.08) respectively. The content of cysteine is equal to that of papain though the content of tyrosine and tryptophan were different as papain has 19 tyrosine and 5 tryptophan. Under the same experimental conditions ribonuclease and lysozyme yielded the reported values.

**Stability**

The enzyme retained its activity between pH 2.0 to 12.0 after prolonged exposure as shown in Fig. 4B. The striking observation is that the enzyme retains its full activity even after prolonged incubation at pH 2.0 for more than a period of one month. However, at pH 1.5 half of the initial activity was lost. Thermostability of the enzyme was also examined by measuring the residual activity after 15 min of incubation at various temperatures as shown in Fig. 5B. Upto 70% of the initial activity was retained at temperatures as high as 70°C. Further, the stability of the enzyme under various conditions, which generally cause loss of activity in the case of other enzymes, was also investigated and it was found that the enzyme is relatively stable. Results of these studies on stability of ervatamin are summarized in Table 3.

**N terminal Sequence**

The N-terminal sequence of the first 21 amino acid

![Graph showing the relative activity vs concentration](image)

**Table 3. Stability of Ertatamin under Various Conditions**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Results*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 2.0</td>
<td>very stable, 100% activity</td>
</tr>
<tr>
<td>8 M urea at pH 7.0</td>
<td>stable, 100% activity</td>
</tr>
<tr>
<td>8 M urea at pH 2.0</td>
<td>42% activity after 24 h</td>
</tr>
<tr>
<td>0.75% SDS</td>
<td>49% activity after 8 h</td>
</tr>
<tr>
<td>4 M GuHCl at pH 7.0</td>
<td>stable, 100% activity</td>
</tr>
<tr>
<td>40% Acetonitrile</td>
<td>stable, 100% activity</td>
</tr>
<tr>
<td>70% Ethanol</td>
<td>stable, 100% activity</td>
</tr>
<tr>
<td>50% Methanol</td>
<td>stable, 100% activity</td>
</tr>
<tr>
<td>Temperature</td>
<td>stable, 100% activity</td>
</tr>
<tr>
<td>(70°C for 15 min)</td>
<td>stable, 100% activity</td>
</tr>
</tbody>
</table>

* Enzyme activity was measured as described in Materials and Methods.

**Fig. 7. Comparison of N-Terminal Amino Acid Sequence of Ertatamin With Known Cysteine Proteases.**

Abbreviations of amino acids follow the alphabetical system.

| ERVATAMIN | LPEQIDWRKXGAVTPVWNQGW |
| PAPAIN    | IPEDWKRQKGVTPVKNQGQS |
| ASCLEPAIN | LPNSIDWRQKNVVFPIKNQGG |
| BROMELAIN | VPSIDWRNYGAVTSVKNNP |
residues of ervatamin has been compared to other known plant cysteine proteinases like papain,\textsuperscript{26} asclepain\textsuperscript{27} and bromelain.\textsuperscript{28} Ervatamin showed considerable sequence similarity with those cysteine proteases. The sequence similarity is shown in Fig. 7.

**Discussion**

A cysteine protease, named as ervatamin, has been isolated and characterized from the latex of *Ervatamina coronaria*. This is the first protease to be isolated from the latex of this valuable plant. Plant sources have yielded some useful endopeptidases and among them papain and bromelain have been used extensively in the food industries and medicine.\textsuperscript{29} Ervatamin, too, seems to be a potential endopeptidase with many interesting properties and may be having some useful applications. Simple and economic purification procedure of ervatamin in good yield, together with the easy availability of the latex, makes the large scale preparation of the protein possible, allowing a look into its various applications along with studies on structure-function relationship.

The estimated molecular mass of ervatamin (25,000 Da) was in the range of molecular mass (20000–35000 Da) reported for other plant cysteine proteinases\textsuperscript{30} and showed proteolytic activity towards natural substrates. However, the activity of ervatamin towards synthetic substrates was very insignificant and little activity was seen towards BAPA, which is an ideal substrate for papain. Thus, specificity of ervatamin for synthetic substrates seems to be different from that of papain.

The proteolytic activity of ervatamin was inhibited by thiol-specific inhibitors but not by serine-specific inhibitors or metal chelators. Ervatamin was maximally activated by various reducing and chelating reagents. All these suggest that the protease belongs to the class of cysteine proteases. This enzyme resists inhibition by proteinaceous inhibitors like STBI, present in protein-rich foods like soybeans. Hence, it may have important implications\textsuperscript{29} in the food industry. The optimum pH of the protease was neutral, which is similar to the optimum pH of papain, chymopapain, and asclepian.

The striking property of ervatamin is its high stability. The enzyme showed almost equal activity, relative to the native form, after prolonged exposure to extreme pH, strong denaturants, temperature, or organic solvents, which are known to inactive enzymes. Ervatamin showed high stability in 8 M urea, 50% methanol, and 70% ethanol, like papain.\textsuperscript{29} However, unlike papain and other reported plant cysteine proteases, ervatamin is stable in 4 M GuHCl and 0.75% SDS even after prolonged periods of exposure. The other remarkable feature of this enzyme is its stability over a broad range of (pH 2.0–12.0) which is unique to best of our knowledge. This extraordinary stability of ervatamin could make it an excellent system for biophysical and crystallographic studies as well.

Analysis of the N-terminal sequence of the first 21 residues of ervatamin and an alignment of the same with those of other plant cysteine proteases showed considerable similarity to papain and bromelain. The amino acid residues common to known plant cysteine proteases were also conserved in the sequence of ervatamin. Sequence similarity between ervatamin and papain is very high, as seen. It thus seems apparent that ervatamin, although obtained from a plant unrelated to papaya (the source of papain), probably shares an ancestral gene with that of papain.

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**References**


